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(57) Abstract

Methods and compositions are provided for *in vitro* long-term hemopoiesis in generating hemopoietic stem cells and hemopoietic progenitors. The methods comprise coculturing mammalian hemopoietic stem cells with substantially pure type 1 endothelial cells, or with type 1 endothelial cell conditioned medium. Without the need for adding exogenous cytokines to the coculture, type 1 endothelial cells maintain and induce proliferation of stem cells, and direct the stem cells to differentiate into hemopoietic progenitor cells.

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COMPOSITIONS AND METHODS FOR INDUCING THE DEVELOPMENT AND DIFFERENTIATION OF HEMOPOIETIC STEM CELLS

FIELD OF THE INVENTION

The present invention relates to a novel discovery that a certain subpopulation of liver endothelial cells can provide the microenvironment necessary for the maintenance and growth of hemopoietic cells. More particularly, the present invention relates to coculturing purified type 1 endothelial cells with primitive hemopoietic precursors (stem cells) in an *ex vivo* method for supporting long-term hemopoiesis by supporting the proliferation and differentiation of the stem cells.

BACKGROUND OF THE INVENTION

Hemopoiesis and Stem Cells

Hemopoiesis (also known as hematopoiesis) is the formation of the various differentiated blood cell lineages (hemopoietic/hematopoietic cells) from precursor cells. Pluripotential or multipotential stem cells can serve as common precursor cells for granulocytes (including neutrophils, eosinophils and basophils), monocytes, erythrocytes, megakaryocytes (and platelets), and lymphocytes. These stem cells, which are present in small numbers in the blood and bone marrow, can both reproduce in forming more stem cells (self renewal) or give rise to the more differentiated hemopoietic cells.

Because it is believed that all mammalian hemopoietic cells could be derived from a single stem cell, stem cells have been used therapeutically to reconstitute a depleted supply of hemopoietic cells *in vivo*. For example, high dose chemotherapy or radiotherapy for treating cancer results in the destruction and resultant depletion of stem cells and more differentiated hemopoietic cells in the bone marrow (aplasia). The depleted cells can be restored by the infusion of stem cells, wherein the stems cells are collected from the patient prior to treatment, or from a

donor. Stem cells have been used to treat marrow aplasia following chemotherapy for breast cancer, ovarian cancer, testicular cancer, brain tumors, neuroblastoma, lymphomas, multiple myeloma, and acute leukemias. Additionally, stem cells are being used to treat aplastic anemia, sickle cell anemia, diabetes, and various autoimmune disorders.

There are previously described methods for generating and releasing more committed progenitor cells from culture of stem cells. For example, hemopoietic stem cells cocultured with stromal cells resulted in stem cell proliferation and differentiation, with stem cells adhering to the multilayer of stromal cells, and the release of more committed progenitor cells in the medium (Gordon et al., 1987, *J. Cell Physiol.* 130:150). Such stromal cells are known to include a mixed cell population comprising reticular cells, macrophages/monocytes, fibroblasts, osteoclasts, adipocytes, and endothelial cells; wherein a minority of the cells are endothelial (Clark and Keating, 1995, *Ann. NY Acad. Sci.* 770:70-80). The stromal layer produces extracellular matrix and mediates direct cellular contact that regulates *in vivo* and *in vitro* hemopoiesis (Wolf et al., 1968, *J. Exp. Med.* 127:205; Trentin, 1971, *Am. J. Pathol.* 65:3; Dexter et al., 1977, *J. Cell Physiol.* 91:335-344). U.S. Patent No. 5,436, 151 discloses a method for the long term culture of mammalian stem cells comprising maintaining the stem cell population (adhered to a substrata) in stromal cell culture medium in a noncontacting relationship. Using such a method, 40-50% of the initial stem cell population can be preserved after 5 weeks. U.S. Patent 5,460,964 disclose the same method, further comprising the addition of cytokines IL-3 and macrophage inflammatory protein-1 alpha (MIP-1 alpha) to maintain self replication and differentiation of the cultured stem cells. Similarly, U.S. Patent No. 5,605,822 discloses a method for *ex vivo* human stem cell proliferation comprising culturing stem cells in the presence of culture medium from stromal

cells, wherein the stromal cells are transformed to produce at least one growth factor.

It is therefore desirable to provide a microenvironment that supports the maintenance and growth of mammalian stem cells, and to provide a microenvironment for sustaining long-term hemopoiesis by supporting the proliferation and differentiation of the stem cells.

SUMMARY OF THE INVENTION

The present invention provides a method for the growth and differentiation of hemopoietic cells in culture. The method comprises maintaining mammalian stem cells, preferably human stem cells, in a coculture with a substantially pure population of type 1 endothelial cells without the need for adding exogenous cytokines to the coculture. In one embodiment of the method, the type 1 endothelial cells provide both a physical matrix and soluble growth factors (cytokines) for maintaining the proliferation and differentiation of stem cells. In this embodiment, the majority of the stem cells in coculture are present in the cell layer adherent to the monolayer of type 1 endothelial cells. By periodically removing stem cells from direct contact with the type 1 endothelial cells in the coculture, a population of self replicating pluripotent stem cells can be maintained.

In addition to maintaining a supply of proliferating stem cells, this embodiment provides for hemopoietic progenitor cells generated from the active hemopoiesis ongoing in the coculture. The type 1 endothelial cells provide a microenvironment that can direct stem cells to differentiate along multiple cell lineages. The majority of the hemopoietic progenitor cells in coculture may be harvested from the non-adherent fraction; i.e., suspended in the medium. By maintaining a supply of proliferating stem cells, and by periodically removing the non-adherent hemopoietic progenitors, active hemopoiesis can

be maintained in culture thereby generating a supply of hemopoietic progenitors.

In another embodiment of the method of the present invention, the stem cells are physically separated from the type 1 endothelial cells, thereby facilitating the harvesting of the stem cells. While there are several ways known to those skilled in the art for achieving such a separation, in one illustration of this embodiment a cell production system is used in which the stem cells and the type 1 endothelial cells are each adhered to separate support matrices. Thus, the supported stem cells are maintained in a non-contacting association with the supported type 1 endothelial cells. In this embodiment, the stem cells are perfused and maintained in the medium from the type 1 endothelial cells ("type 1 endothelial cell conditioned medium") during culturing or coculturing. By using the method illustrated in this embodiment, a population of self replicating pluripotent stem cells can be maintained. This embodiment also provides for hemopoietic progenitor cells generated from the active hemopoiesis ongoing in the coculture. The majority of the hemopoietic progenitor cells may be harvested from the non-adherent fraction; i.e., from the medium.

These and further features and advantages of the invention will be better understood from the description of the preferred embodiments when considered in relation to the figures in which:

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph illustrating the ability of either unactivated type 1 endothelial cells or unactivated type 2 endothelial cells to support hemopoiesis *in vitro*.

FIG. **2A** is a photograph showing hemopoietic cell formation on type 1 endothelial cells after 5 days of coculture.

FIG. 2B is a photograph showing hemopoietic cell formation and active hemopoiesis on type 1 endothelial cells after 3 weeks of coculture.

FIG. 3A is a graph illustrating the number of erythroid progenitors present in either coculture of unactivated type 1 endothelial cells or unactivated type 2 endothelial cells.

FIG. 3B is a graph illustrating the number of granulocyte/ macrophage progenitors present in either coculture of unactivated type 1 endothelial cells or unactivated type 2 endothelial cells.

FIG. 3C is a graph illustrating the number of megakatyocyte progenitors present in either coculture of unactivated type 1 endothelial cells or unactivated type 2 endothelial cells.

FIG. 4A is a bar graph illustrating the cytokine expression of unactivated type 1 endothelial cells.

FIG. 4B is a bar graph illustrating the cytokine expression of unactivated type 2 endothelial cells.

FIG. 4C is a bar graph illustrating the cytokine expression of type 1 endothelial cells activated by TNF-α.

FIG. 4D is a bar graph illustrating the cytokine expression of type 2 endothelial cells activated by TNF-α.

FIG. 5 is a bar graph illustrating the comparison of nitric oxide production (measured as NO_2) in unactivated type 1 endothelial cells, unactivated type 2 endothelial cells, and type 1 endothelial cells or type 2 endothelial cells activated by either TNF-α, or IFN-γ, or by a combination of TNF-α and IFN-γ.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

The term "coculture" is used herein, for purposes of the specification and claims, to mean either the culture of stem cells in the physical presence of substantially pure type 1 endothelial cells and growth factors secreted from the type 1 endothelial cells, or the culture of stem cells in the presence of type 1 endothelial cell conditioned

medium containing growth factors secreted from the type 1 endothelial cells (e.g., medium harvested from a type 1 endothelial cell culture; therefore lacking the type 1 endothelial cells themselves).

The term "hemopoietic progenitor" is used herein, for purposes of the specification and claims, to mean a lineage-committed hemopoietic cell that has differentiated from a stem cell; e.g., a heterogenous mixture of cells at various stages of differentiation and maturation along the myeloid, erythroid, and megakaryocyte lineages. Hemopoietic progenitors may include granulocyte-monocyte colony forming cells (GM-CFC), myeloblasts, promonocytes, and promyelocytes, as cells committed to the monocyte and/or neutrophil lineages; eosinophil colony forming cells (EO-CFC), myeloblasts, and myelocytes, as cells committed to the eosinophil lineage; erythrocyte colony forming cells, pronormoblasts, and normoblasts, as cells committed to the erythrocyte lineage; megakaryocyte colony forming cells, megakaryoblasts, and promegakaryocytes, as cells committed to the megakaryocyte/platelet lineage; and lymphoid stem cells, as cells committed to the lymphocyte lineages.

The term "type 1 endothelial cell" is used herein, for purposes of the specification and claims, and as more apparent by the detailed description that follows, to mean a subpopulation of endothelial cells that are found *in vivo* to predominate in the periportal segment of the organ sinusoids. Such organs may include liver, lung, brain, lymph node, bone marrow, and adrenal gland. Type 1 endothelial cells may include primary cloned lines (e.g., by limiting dilution and maintenance in culture), or immortalized (transformed) cells. The type 1 endothelial cells may be mammalian in origin, and in a preferred embodiment, human in origin. Additionally, if desired, the type 1 endothelial cells may be allogenic or autologous with respect to the hemopoietic stems cells cocultured therewith.

In the development of the present invention it was demonstrated that type 1 endothelial cells survive for at least several weeks in culture, in addition to maintaining a stable phenotype after multiple passages (greater than 10 passages). Thus, while not necessary to practice the present invention, type 1 endothelial cells may be immortalized for use in the present method. There are several methods known to those skilled in the art for immortalizing endothelial cells, such as by the use of viral or cellular oncogenes (see, e.g., review by Punchard et al., 1994, *Biochem. Soc. Trans.* 22:197S). For the immortalized type 1 endothelial cell line to be useful in the method of the present invention, it would have to retain the cell markers and cytokine secretion characteristic of type 1 endothelial cells, as more defined herein. Various viral oncogenes have been used to transform endothelial cells resulting in immortalized endothelial cells showing a prolonged life span, and retaining endothelial cell characteristics including the secretion of cytokines. These can include transformation with SV40 T-antigen (Moldovan et al., 1996, *In Vitro Cell Dev. Biol. Anim.* 32:16-23; Yamazaki et al., 1995, *In Vivo* 9:421-426; Nakano et al., 1995, *Biochem. Mol. Biol. Int.* 36:715-722); transformation with the E1A adenovirus gene (Roux et al., 1994, *J. Cell Physiol.* 159:101-113); and transformation with the E6/E7 genes of human papilloma virus (Fontijn et al., 1995, *Exp. Cell Res.* 216:199-207) (all herein incorporated by reference).

The term "substantially pure" is used herein in conjunction with type 1 endothelial cell, for purposes of the specification and claims, to mean either a heterogeneous population of cells in which type 1 endothelial cells comprise greater than or equal to approximately 70% of the cells comprising the heterogenous population (a percentage greater than any individual or collective endothelial cell populations in bone marrow stromal cells); or a homogeneous population of type 1 endothelial cells wherein the type 1

endothelial cells comprises greater than 90% of the cell population. The term "stem cells" is used herein for purposes of the specification and claims, to mean pluripotential or multi-potential cells that can serve as common precursor cells for hemopoietic progenitors. Preferably the stem cells are mammalian stem cells. In a preferred embodiment, the mammalian stem cells are human stem cells. Mammalian stem cells have been characterized by the expression of various cell surface markers, and by function. Hemopoietic stem cells have been identified, and purified (e.g., by fluorescent-activated cell sorting), on the basis of the expression or lack of expression of cell surface markers including lineage marker negative (Lin(-)); CD34(+); and stem cell antigen (Sca-1(+)). Other cell surface markers used for the identification of stem cells may include HLA-DR (-) and c-kit receptor (+) (see, e.g., Nishio et al., 1996, *Stem Cells* 14:584-591; Messner, 1991, *Bone Marrow Transplant* 7 (Suppl): 18-22).

The stem cells may be allogeneic or autologous with respect to the intended recipient. For example, autologous transplants of stem cells effect complete remission in patients with multiple myeloma in up to 50% of the cases; whereas the complete response rate for allogeneic transplants ranges from 30-50% (Jagannath et al., 1994, *Oncology* 8:89-103). The source of the stem cells may include bone marrow, cord blood, or peripheral blood. Cord blood has been shown to be a source for pluripotential stem cells (see, e.g., Fishman et al., 1992, *Immunol. Lett.* 34:189-193). Compared to other sources, cord blood appears to have a higher stem cell concentration. Additionally, harvesting stem cells from bone marrow is painful. Another advantage of using cord blood is that there appears to be a lower risk of graft versus host disease (*Cancer Weekly Plus*, December 23, 1996). Methods for collection and purification of stem cells are known in the art (Visser and Van Bekkum, 1990, *Exp. Hematol.* 18:248-256; Verfaillie et al., 1990, *J. Exp.*

Med. 172:509,; herein incorporated by reference). Human stem cells from peripheral blood may be collected by cytapheresis during the recovery from short-term aplasia induced by drug treatment (such as with chemotherapeutic agents or sargramostim), wherein the drug-induced stem cell mobilization comprises an expansion of the circulating pool of stem cells (Fermand and Brouet, 1995, *Annu. Rev. Med.* 46:299-307; see also, Nishio et al., 1996, *supra*).

Stem cells may also comprise stem cells transfected with an expression vector. It has been reported that transfected stem cells can replicate (*Bioventure View*, 1996, Vol. 1, No. 2) and generate persistent populations of differentiated cells expressing the transgene long-term in various tissues (*Vaccine Weekly*, 1996, February 26). In that regard, a Phase I *ex vivo* study has been completed in which patients with chronic granulomatous disease (CGD) received infusions of autologous stem cells that were transfected with copies of the normal gene for NADPH oxidase (*Bioventure View, supra*) after *in vitro* studies and an *in vivo* animal model system showed efficacy of the gene therapy in restoring phagocyte oxidant-dependent host function (Weil et al., 1997, *Blood* 89:1754-61; Mardiney et al., 1997, *Blood*, 89:2268-75). Methods and vectors for transfecting stem cells are known in the art (Weil et al., 1997, *supra*; Mardiney et al., 1997, *supra*; see also European Patent disclosure WO 96/08560: Hematopoietic stem cells transfected with adeno-associated viral vectors for cellular gene therapy; herein incorporated by reference).

The terms "preferentially expressed by type 1 endothelial cells" is used herein, for purposes of the specification and claims, to mean a molecule expressed on the surface of type 1 endothelial cells, wherein the level of expression (measured directly or indirectly, and including by presence or by activity) of such molecule is at least 3 to 4 times that expressed by other subpopulations of endothelial cells contained in the sinusoidal area of an

organ. Thus, preferential expression may include detection of expression of the molecule on or by type 1 endothelial cells, and absence of detection of the same molecule on or by type 2 endothelial cells; or a log greater expression of the same molecule on or by type 1 endothelial cells as compared to expression on or by type 2 endothelial cells.

In one embodiment of the present invention, a method is provided for preserving and inducing proliferation of stem cells by maintaining the stem cells in a coculture with a substantially pure population of type 1 endothelial cells (a) without the need for adding exogenous cytokines to the coculture; and (b) in direct contact with the type 1 endothelial cells. In this embodiment, the type 1 endothelial cells provide both a physical matrix and soluble growth factors (cytokines) for supporting the maintenance and proliferation of stem cells. A monolayer of substantially pure type 1 endothelial cells is preformed and adhered onto the surface of a vessel, such as a reactor, followed by the addition of stem cells to the culture resulting in a coculture promoting the direct contact between the stem cells and the preformed monolayer of type 1 endothelial cells.

The coculture is then incubated in conditions (with culture medium and incubator temperatures) suitable for promoting the maintenance and proliferation of stem cells ("physiological acceptable conditions"). Typically, serum supplemented or serum-free substitute supplemented standard tissue culture medium may be used, with incubation at temperatures around 37°C. As is with standard tissue culture, maintenance of the cell coculture will require the introduction of nutrients and the removal of metabolic waste products (typically achieved by periodically removing culture medium and adding fresh medium), and by maintaining the appropriate atmospheric conditions. Although, it is

appreciated by those skilled in the art that the incubation conditions can be varied.

The proliferating stem cells can be gently removed (by enzymatic or physical means) from the type 1 endothelial cells, allowing for the harvesting of stem cells and thereby providing additional space for continued stem cell proliferation. For example, physical means for separating cells of different types may be achieved by their differential tolerance to shear stress (see, e.g. U.S. Patent No. 5,605,822). Alternatively, enzymatic conditions (such as trypsin concentration and time of incubation in trypsin) may be utilized to remove the stem cells while substantially maintaining the integrity of the type 1 endothelial cell monolayer. It may be desirable to analyze or further purify the harvested stem cells before infusing a therapeutically effective amount of the harvested stem cells. Use and efficacy of stems cells for transplantation to effect hemopoietic reconstitution after ablative chemotherapy has been described previously (see, e.g., Pierelli et al., 1994, *Br. J. Haematol.* 86:70-5).

In addition to maintaining a supply of proliferating stem cells, this embodiment provides for hemopoietic progenitors generated from the active hemopoiesis ongoing in the coculture. The type 1 endothelial cells provide a microenvironment that can direct stem cells to differentiate along multiple cell lineages. *In vitro*, type 1 endothelial cells tend to select for differentiation at early stages of hemopoiesis resulting in the accumulation of hemopoietic progenitors, and thereby tends to restrict the accumulation of mature hemopoietic cells. This tendency has several attendant advantages. For example, even with the infusion of mobilized stem cells, engraftment time is 8 days on average. Engraftment time is the time necessary for the stem cells to find, and implant themselves in, the bone marrow. However, engraftment marks only the beginning of recovery of bone marrow function. The stem cells need to

grow and differentiate for some time before an individual's immune system, destroyed by chemotherapy or radiotherapy, can be reconstituted. Because it takes a while for the peripheral blood cell populations to be reconstituted, this time period leaves the individual at risk for infection, as well as susceptible to other morbidity associated with relatively depleted peripheral blood cell populations.

Thus, among the *in vitro* and *in vivo* uses, hemopoietic progenitors generated and cultivated from the method of the present invention may be suitable for transfusion into recipients (autologous or allogeneic). Such a transfusion may reduce the morbidity associated with relatively depleted peripheral blood cell populations by accelerating the hematological and immunological reconstitution of the relatively depleted populations. This is because it is believed that *in vivo* the hemopoietic progenitors can multiply and differentiate into the mature, highly specialized cells constituting the peripheral blood cell populations including red blood cells (for carrying oxygen throughout the body); platelets (for blood clotting); and lymphocytes, granulocytes (basophils, eosinophils, neutrophils) and macrophages/monocytes (for disease-fighting and immune system functions). It is also believed that the hemopoietic progenitors may tend to be more "naive" (like stem cells) to the immune system, than the mature hematopoietic cells. Additional advantages attendant to the hemopoietic progenitors include that they can be frozen and stored for long periods of time; and, if autologous, can obviate the need for cross-matching and immune sensitization, and risk of blood-borne infections, as compared to use of allogeneic cells. As with any transfusion or infusion, a careful monitoring of the recipient and donor should be considered to watch for side effects.

The majority of the hemopoietic progenitors in coculture may be harvested from the non-adherent fraction; i.e., as present in the medium from the reactor. The

hemopoietic cells can be harvested by removing the medium from the coculture, and separating the medium from the cells present, such as by physical means. Such physical means may include, but is not limited to, filtration, chromatography, or centrifugation. It may be desirable to analyze or further purify the harvested hemopoietic progenitors before transfusing a therapeutically effective amount of the harvested cells. By maintaining a supply of proliferating stem cells, and by periodically removing the non-adherent hemopoietic progenitors, active hemopoiesis can be maintained in culture thereby generating a supply of hemopoietic progenitors.

In another embodiment of the method of the present invention, the stem cells are cocultured in physical separation from the substantially pure type 1 endothelial cells, thereby facilitating the harvesting of the stem cells. Also, by maintaining a supply of proliferating stem cells, and by periodically removing the non-adherent hemopoietic progenitors, active hemopoiesis can be maintained in culture thereby generating a supply of hemopoietic progenitors. Thus, the type 1 endothelial cell conditioned medium provides necessary growth factors for maintenance and proliferation of stem cells in culture. While there are several ways known to those skilled in the art for achieving such a separation, in one illustration of this embodiment a cell production system is used in which the stem cells and the type 1 endothelial cells are each adhered to separate support surfaces; whether it is at least two separate support matrices, or one support matrix having on one side the type 1 endothelial cells and having on the other side the stem cells. There are many different proteins or compositions known to those skilled in the art to facilitate hemopoietic cell adhesion and attachment to a support matrix. These include, but are not limited to, extracellular matrix, collagen, vitrogen, entactin, nidogen,

glycosaminoglycans, proteoglycans, laminin, fibronectin, synthetic peptides, and combinations thereof.

A commercially available cell culture system that is suitable for, and illustrates, the use of two separate support matrices is the Transwell™ system (Corning Costar Corp., Cambridge, MA). In this system, two cell culture chambers are provided, wherein a first chamber fits inside a larger second chamber. The type 1 endothelial cells may be added to the inner surface forming the bottom of the second chamber. The stem cells may be added to the inner surface forming the bottom of the first chamber wherein that bottom comprises a microporous membrane. The microporous membrane is prepared such that it facilitates adhesion and attachment of the stem cells, and provides pores of sufficient size (e.g., 0.4 to 0.5 micro m) to allow for transport of culture medium therethrough but prevent the passage of cells therethrough. Thus, the membrane allows for physical separation (i.e., in a non-contacting association) between the type 1 endothelial cells and the stem cells, while allowing for the stem cells to be perfused in and maintained by the medium from the type 1 endothelial cells in coculture. A similarly functioning cell culture device and its operation is also described in U.S. Patent No. 5,605,822 (herein incorporated by reference). U.S. Patent No. 5,605,822 also illustrates the mode of this embodiment wherein one support matrix is utilized in coculture to maintain the type 1 endothelial cells and the stem cells in a non-contacting association. In any of the above-described embodiments, the liquid growth medium in the coculture may be in simultaneous contact with both the type 1 endothelial cells and the stem cells, wherein the medium is exchanged at fixed intervals thereby enhancing the maintenance of the coculture. Alternatively, and as in an embodiment wherein the substantially type 1 endothelial cells and the stem cells are maintained in separate cell culture chambers, type 1 endothelial cell conditioned medium may be controllably circulated into, and

may be exchanged for existing medium in, the chamber containing the stem cells thereby perfusing the stem cells, and wherein the medium is exchanged at fixed intervals thereby enhancing the maintenance of the coculture. Commercially available bioreactor systems that may be useful in practice of the method of the present invention include systems marketed by Aastrom Biosciences Inc. (Ann Arbor, Michigan).

EXAMPLE 1

This example illustrates isolation of type 1 endothelial cells found in the unique capillary zones in organ sinusoids, and identification of this cell sub-population as an endothelial cell subpopulation. The sinusoidal walls of certain organs are composed of at least two kinds of endothelial cells. For example in the liver, type 1 endothelial cells are located proximal to the peri-portal side of the liver sinusoids. In contrast, type 2 endothelial cells are located proximal to the perihepatic side of the sinusoids. Type 1 endothelial cells were isolated and purified as essentially taught by Vidal-Vanachlocha et al. (1993, *supra*, herein incorporated by reference). Basically, a portion of sinusoidal tissue from the organ in which is enzymatically treated (e.g., pronase E (0.02%), type 1 collagenase (0.05%), and DNase (0.03%)) to achieve a cell suspension. The cell suspension is filtered through a nylon screen, washed and centrifuged several times, and treated (17.5% metrizamide) to remove cell debris and erythrocytes. After resuspension in a physiological buffer, an enriched population of type 1 endothelial cells was achieved by counterflow elutriation at 10°C and 2,400 rpm with a flow rate of approximately 37 ml/minute. The collected cell fraction contained essentially type 1 endothelial cells (>90% of endothelial cells) together with Kupffer cells in a proportion of about 2:3. Further purification of the type 1 endothelial cells was achieved on

discontinuous arabinogalactin density gradient centrifugation (containing densities of 1.02, 1.03, 1.04, and 1.06) at 20,000 rpm for 30 minutes at 25°C. Cells sedimenting in the 1.04 to 1.06 interphase were exclusively type 1 endothelial cells as characterized by more than 90% high-wheat germ agglutinin (WGA) binding. Using this procedure, for example, a yield of at least 4 x 10^6 type 1 endothelial cells can be obtained from 8 grams of organ tissue. Yields of type 1 endothelial cells may be improved by using the portal vein as tissue from which the cells are isolated, consistent with the anatomy of the location of type 1 endothelial cells.

It is noted here that it will be apparent from the further characterization of type 1 endothelial cells that follows, isolation and purification of type 1 endothelial cells may be facilitated at any stage by using one or more targeting molecules having affinity for cell surface receptors preferentially expressed by type 1 endothelial cells (as compared to expression by other cells in the area) as an immobilized affinity molecule in a process of affinity chromatography. The process of affinity chromatography is a standard technique known to those skilled in the art. As an alternative, and using a combination of the targeting molecules having affinity for cell surface receptors preferentially expressed by type 1 endothelial cells (as compared to expression by other cells in the area), fluorescent activated cell sorting may be used to prepare a population of substantially pure type 1 endothelial cells. The process of fluorescent activated cell sorting is a standard technique known to those skilled in the art.

To identify and confirm that the isolated subpopulation comprising type 1 cells was endothelial cell in nature, subpopulations of type 1 endothelial cells and of type 2 endothelial cells were isolated and purified using a method outlined above, and evaluated for expression of endothelial cell markers by nucleic acid amplification using

commercially available primers specific for the endothelial cell markers. The endothelial cell markers included stem cell factor (SCF, see e.g., Koenig et al., 1994, *Blood* 83:2836-43), vascular endothelial cell growth factor receptor flt-1 (see, e.g., Mochida et al., 1996, *Biochem. Biophys. Res. Commun.* 226:176-9), von Willebrand factor (VW, see Table 2 herein; Gendron et al., 1996, *Dev. Biol.* 177:332-46), leukocyte inhibitory factor (LIF), and CD34 (Couvelard et al., 1996, *Blood* 87:4568-80). Other markers, that are not normally expressed in detectable levels in endothelial cells and which were evaluated as a control, included transforming growth factor beta (TGF-β), erythropoietin (EPO), interleukin-3 (IL-3), erythropoietin (EPO), macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), and vascular endothelial cell growth factor (VEGF). The results of the characterization of subpopulations of type 1 endothelial cells and of type 2 endothelial cells are shown in Table 1.

Table 1

Cell marker	type 1 endothelial cells	type 2 endothelial cells
SCF	+	+
FLT-1	+	+
LIF	+	+
CD34	+	+
TGF-β	-	-
EPO	-	-
G-CSF	-	-
M-CSF	-	-
IL-3	-	-
VEGF	-	-

Table 1 illustrates that certain cell markers (e.g., cytokines) expressed by endothelial cells are expressed by the subpopulation of type 1 cells thereby confirming that this subpopulation is of endothelial cell type.

EXAMPLE 2

This example illustrates characterization of unactivated type 1 endothelial cells and unactivated type 2 endothelial cells in analyzing if these endothelial cells subpopulations can provide some or all of the various factors necessary for the maintenance and growth of circulating hemopoietic stem cells during extramedullary hemopoiesis. As will be more apparent from the following characterization, circulating hemopoietic stem cells must first arrest in the organ. Such arrest appears to be due a local environment provided by type 1 endothelial cells, e.g. by expression of endothelial cell adhesion molecules or other cell surface receptors which (a) are preferentially expressed in higher amounts by type 1 endothelial cells (as compared to other cell populations in the sinusoids of the organ), and (b) initially mediate the colonization of the seeding hemopoietic stem cell to the endothelium comprising the hematopoietic environment.

Cell Surface Marker Expression

For characterization and comparison, subpopulations of type 1 endothelial cells (predominate in the periportal segment of the organ sinusoids) and of type 2 endothelial cells (predominate in the perivenous segment of the organ sinusoids) were isolated and purified using the methods according to Example 1. Each subpopulation was evaluated for expression of endothelial cell surface markers including von Willebrand factor (VW), N-acetylglucosamine (GlcNAC), N-acetyl galactosamine (GalNAC), mannose receptor, receptors having albumin affinity, intracellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion

molecule-1 (VCAM-1), platelet cell adhesion molecule-1 (PCAM-1), major histocompatibility antigen-II (MHC-II); and phagocytic ability. VW was measured by immunofluorescence using a commercially available rabbit anti-human factor VIII antigen antibody. Essentially, a monolayer of either type 1 endothelial cells or type 2 endothelial cells were fixed with acetone-methanol-40% formalin, followed by washing with PBS. The cultures were incubated with the anti-human factor VIII antigen antibody for 40 minutes at 4°C. Fluorescence was measured by quantitative fluorescence microscopy (+ = detectable fluorescence; - = absence of detectable fluorescence).

GlcNAC was measured by binding specificity with the lectin wheat germ agglutinin (WGA). Viable type 1 endothelial cells and type 2 endothelial cells were separately assayed for GlcNAc expression by flow cytometry. First, each subpopulation was incubated *in vitro* at 37°C for 5 minutes in the presence of fluorescein isoythiocyanate-labelled wheat germ agglutinin lectin (FITC-WGA, 10µg/ml). Flow cytometric analysis was then performed using an argon laser, tuned at 488 nm, 250 mW, with a nozzle tip having an 80mm diameter. Cells in each sample were simultaneously measured for forward light scatter, side scatter, and green fluorescence and red fluorescence emissions. The data was stored and analyzed using standard methods of analysis (see for example, Vidal-Vanachlocha et al. 1993, *supra*). The degree of lectin binding (and hence the degree of GlcNAC expression) was determined by the intensity of fluorescence and measured by semiautomatic evaluation. Alternatively, the degree of lectin binding was measured by quantitative fluorescence microscopy. "Very high" expression of GlcNAC, as compared to "very low" expression of GlcNAC, reflects at least a 4 fold to 6 fold increase in intensity of fluorescence.

Using methods similar to those used for measuring GlcNAC, GalNAC was measured by binding with lectin having

specificity for GalNAC (e.g., *Dolichos biflorus*, *Vicia villosa*, *Wisteria floribunda*, *Sophora japonica*, peanut lectin, etc.; wherein + = detectable fluorescence; - = absence of detectable fluorescence). Using methods similar to those used for measuring GlcNAC, mannose receptor was measured by incubation of the respective cell subpopulations with fluorescence-labeled ovalbumin (FITC-conjugated OA), and measurement by quantitative fluorescence microscopy of endocytosed FITC-conjugated OA which is proportional to mannose receptor expression (wherein high = visible fluorescence; and low = absence of visible fluorescence).

Endothelial cells contain membrane-associated polypeptides that bind Amadori-modified glycated albumin (GA), the predominant form of in which nonenzymatically glycated albumin exists *in vivo*, but do not bind to unmodified albumin. Viable type 1 endothelial cells and type 2 endothelial cells were separately assayed for expression of receptors having albumin affinity by flow cytometry. First, each subpopulation was incubated *in vitro* at 37°C for 5 minutes in the presence of fluorescein isoythiocyanate labelled Amadori-modified glycated albumin (FITC-GA, 10μg/ml). The cell subpopulations were then analyzed using flow cytometric analysis as described above (wherein high = detectable fluorescence; and low = absence of detectable fluorescence).

Endothelial cell adhesion molecules, and the integrin family to which they bind, are important for the colonization of hematopoietic cells, homing of lymphocytes, and proliferation of lymphocytes. Viable type 1 endothelial cells and type 2 endothelial cells were separately assayed for expression of ICAM-1, VCAM-1, and PCAM-1 by flow cytometry. First, each subpopulation was incubated *in vitro* at 4°C for 40 minutes in the presence of saturating doses of either fluorescein isoythiocyanate labelled mouse monoclonal antibody against ICAM-1 (FITC-anti-ICAM-1), FITC-labelled anti-VCAM-1, or FITC-labelled anti-PCAM-1. The monoclonal

antibodies used are commercially available. The cell subpopulations were then analyzed using the flow cytometric methods, data collection, and analysis as outlined above. For ICAM expression, "very high" represents a log of fluorescence intensity (e.g., 10^2) greater than "low" expression (between 10^0 and 10^1). For VCAM, the expression by type 1 endothelial cells was similar to that by type 2 endothelial cells ("high to very high" is 10^1 to 10^2 log fluorescence intensity). For PCAM-1, (-) correlates with absence of detectable expression.

Major histocompatibility antigen-II (MHC-II) was measured by flow cytometry using fluorescent labeled monoclonal antibodies having binding specificity to MHCII. For MHC-II, (-) means absence of detectable expression. The ability of the respective endothelial cell subpopulations to engulf latex beads by phagocytosis was evaluated by utilizing commercially available fluorescent beads (0.5 micrometers in diameter) and flow cytometry to determine bead uptake as well as the number of beads per cell (see, e.g., Dunn and Tyrer, 1981, *J. Lab. Clin. Med.* 98:374-381; Doolittle, 1987, *Hepatology* 9:696-703). For phagocytic ability, (+) means presence of detectable activity; and (-) means absence of detectable activity.

The results of the characterization and comparison of cell surface molecules expressed by unactivated type 1 endothelial cells and by unactivated type 2 endothelial cells are shown in Table 2. Table 2 illustrates that certain cell surface molecules preferentially expressed on unactivated type 1 endothelial cells may be responsible for the arrest and/or survival of any hemopoietic stem cells which may subsequently attempt to seed the organ.

Table 2

Error! Bookmark not defined.Cell surface molecule	type 1 endothelial cells	type 2 endothelial cells
VW	+	+
GlcNAC	very high	very low
mannose receptor	high	low
affinity for GA	high	low
phagocytic ability	+	-
ICAM-1	very high	low
VCAM-1	high	very high
PCAM-1	-	-
MHC-II	-	-
GalNAC	+	+

Cytokine Expression

Cell surface receptors preferentially expressed by type 1 endothelial cells may contribute to initially mediating the colonization of the seeding hemopoietic stem cell to the endothelium comprising the hematopoietic environment. However, as will be more apparent from the following characterizations of proteins/factors expressed by type 1 endothelial cells, growth factors secreted by type 1 endothelial cells interact directly with hemopoietic stem cells for the colonized hemopoietic stem cells to subsequently develop into hematopoietic foci. Subpopulations of unactivated type 1 endothelial cells and of unactivated type 2 endothelial cells were characterized and compared for cytokine expression. Each subpopulation was isolated and purified using the methods according to Example 1, and then evaluated for the presence (+) or absence (-) of detectable expression of cytokines including stem cell factor (SCF), interleukin-1β (IL-1β), interleukin-3 (IL-3),

interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10), erythropoietin (EPO), thrombopoietin (TPO), macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), transforming growth factor beta (TGF-β), and interferon gamma (IFN-γ). The cytokine expression was evaluated by commercially available enzyme linked immunosorbent assays (ELISA) and performed using standard methods known to those skilled in the art (see, e.g., Endo et al., 1996, *Res. Commun. Mol. Pathol. Pharmacol.* 94:23-38).

The results of the characterization and comparison of cytokines expressed by unactivated subpopulations of type 1 endothelial cells and by unactivated type 2 endothelial cells are shown in Table 3.

Table 3

Cytokine	type 1 endothelial cells	type 2 endothelial cells
SCF	+	+
IL-1β	+	-
IL-3	-	-
IL-4	-	-
IL-6	-	+
IL-10	+	+
EPO	-	-
TPO	+	+
M-CSF	-	-
G-CSF	-	-
GM-CSF	+	-
TGF-β	-	-
IFN-γ	+	+

Table 3 illustrates that certain cytokines expressed on unactivated type 1 endothelial cells play a role in the survival of hemopoietic stem cells after arrest in a hematopoietic microenvironment. More particularly, both IL-1β and GM-CSF secreted by type 1 endothelial cells may play a role in maintenance of arrested hemopoietic stem cells in the hematopoietic microenvironment. For example, GM-CSF can stimulate a single bone marrow stem cell to proliferate and differentiate into mature neutrophils, eosinophils, granulocytes, or macrophages (see for example, Fan et al., 1991, *In Vivo*, 5:571-7). It has been reported that IL-3 supports terminal differentiation of hemopoietic progenitors to mature cells (Valent et al., 1990, *Blut* 61:338-45). From these results it is noted that type 1 endothelial cells do not express detectable levels of IL-3, which may be one reason why *in vitro* type 1 endothelial cells tend to select for differentiation at early stages of hemopoiesis and tends to restrict the accumulation of mature hemopoietic cells.

EXAMPLE 3

The mechanisms for extramedullary hematopoiesis are not well defined. For example, the liver is an important hematopoietic organ during fetal life; however, liver hematopoiesis ceases after birth. From an *in vivo* experimental model of extramedullary hematopoiesis, it appears that the hematopoietic microenvironment components are probably expressed in the liver sinusoids (Barberá-Guillem et al., 1989, *Hepatology* 9:29-36). A hematopoietic microenvironment is known to those skilled in the art as a complex system composed mainly of stromal cells comprising fibroblasts, endothelial cells, adipocytes, osteoclasts, and monocytes. The stromal cells secrete cytokines, produce extracellular matrix and mediate direct cellular contact, and perform other functions which contribute to *in vivo* and *in vitro* hematopoiesis (Wolf et al., 1968, *J. Exp. Med.*

127:205; Trentin et al., 1971, *Am. J. Pathol.* 65:3; Dexter et al., 1977, *J. Cell Physiol.* 91:335-44). While the capacity of bone marrow stromal cells to support and regulate hematopoiesis has been demonstrated, the hematopoietic microenvironment in organs other than bone marrow remain undefined. In particular, the unknowns include which cell populations are necessary to provide the *in vivo* hematopoietic microenvironment in organs other than bone marrow; how the cells support and regulate such a hematopoietic microenvironment; and whether such cells can support *in vitro* long-term hemopoiesis. This example illustrates that it is the type 1 endothelial cells, and not type 2 endothelial cells or other cell subpopulations, in the sinusoids of organs that represent the main element of the hematopoietic microenvironment. Further, it is demonstrated herein that type 1 endothelial cells maintain and induce proliferation of stem cells in a process of supporting *in vitro* long-term hemopoiesis which generates hemopoietic progenitors.

Unactivated type 1 endothelial cells and unactivated type 2 endothelial cells were evaluated for their ability to support hematopoiesis *in vitro* by providing the microenvironment necessary for the maintenance and growth of hemopoietic cells. Type 1 endothelial cells and type 2 endothelial cells were isolated from murine liver using the methods according to Example 1. To obtain pure endothelial cell subpopulation cultures, type 1 endothelial cells and type 2 endothelial cells were each cloned by limiting dilution. There are several methods known to those skilled in the art to isolate and purify hemopoietic stem cells from various tissues (see, e.g., review by Visser and Van Bekkum, 1990, *Exp. Hematol.* 18:248-256). In this illustration, donors were pretreated with an agent (e.g., vinblastine, 5-fluorouracil, or hydroxyurea) to result in relative enrichment for the stem cells. More particularly, pluripotent hemopoietic stem cells were isolated from mice

by pre-treating mice with 5-fluorouracil (5-FU) to increase the yield of stem cells; followed by isolation of Lin-, Sca+ cells (Nishio et al., 1996, *supra*; McNiece et al., 1990, *Int. J. Cell Cloning* 8:146-160; and Ogawa et al., 1991, *J. Exp. Med.* 174:63-71). Briefly, the animals were pre-treated with a single intraperitoneal injection of 5-FU at 150 mg/kg body weight. Bone marrow cells were harvested two days post-injection. This population of bone marrow cells includes primitive multipotent precursors which are uncommitted cells expressing very early hemopoietic stem cell markers such as Sca, and the c-kit receptor, while having no detectable expression of Lin. This population of bone marrow cells was resuspended in cell culture medium (Iscove's modified Dulbecco's medium) supplemented with 20% fetal calf serum and cultured in tissue culture dishes for 1 hour to remove adherent cells. The non-adherent bone marrow cells were removed and resuspended in the cell culture medium at $5x10^5$ cells/ml. These non-adherent cells represent a population of hemopoietic stem cells from 5-FU treated donors.

Additionally, hemopoietic stem cells (Lin(-), Sca(+) cells) were isolated from normal mice (i.e., not treated with 5-FU) by immunomagnetic separation according to previously described methods (Spangrude et al., 1988, *Science* 241:58; 1990, *Exp. Hematol.* 18:920-23). Briefly, the bone marrow cells were incubated with a cocktail of antibodies having binding specificities for cell surface receptors including B220, GR-1, MAC-1, Lyt-2 (CD8), Ly-1 (CD5), L3T4 (CD4), and TER119. After washing the incubated cells, sheep anti-rat IgG(Fc) conjugated-immunomagnetic beads (commercially available) were added, and the mixture was incubated for 4°C for 45 minutes. Cells bound to the magnetic beads were removed with a magnetic particle concentrator. Lin(-) cells recovered from the supernatant were incubated with either fluorescein isothiocyanate-

conjugated rat anti-mouse Ly-6A/E antibody, or an isotype-matched control antibody. The cells were washed, and Lin (-), Sca(+) cells were isolated by cell sorting using a cell sorter. The final recovery of Lin(-), Sca(+) cells from unfractionated bone marrow cells was approximately 0.05% with a purity greater than 95%.

Having an isolated and purified subpopulation of type 1 endothelial cells, an isolated and purified subpopulation of type 2 endothelial cells, and two isolated and purified subpopulations of hemopoietic stem cells (one subpopulation from 5-FU treated mice, and one subpopulation from untreated mice), analyzed was the ability of either type 1 endothelial cells or type 2 endothelial cells to support *in vitro* hemopoiesis. That is, type 1 endothelial cells and type 2 endothelial cells were each tested for ability *in vitro* to maintain and to induce proliferation, in the absence of exogenous cytokines, of hematopoietic stem cells isolated from 5-FU treated mice. Separate cultures of cloned type 1 endothelial cells, and of cloned type 2 endothelial cells, were maintained in cell culture medium (Iscove's modified Dulbecco's medium supplemented with 20% fetal calf serum). Hemopoietic stem cells isolated from 5-FU treated mice were inoculated ($1x10^4$ cells/well) onto a confluent monolayer of type 1 endothelial cell culture or type 2 endothelial cell culture. Collagen-precoated control wells, to which the stem cells were added, included those containing tissue culture medium alone, or tissue culture medium with a combination of cytokines (stem cell factor (SCF) at 50ng/ml; IL-3 at 1ng/ml; IL-6 at 10ng/ml; and erythropoietin (EPO) at 5U/ml). For each of the cocultures, half of the medium was collected every 5-7 days and replaced with the respective fresh medium.

The number and viability of non-adherent cells present in the respective collected coculture medium were counted with a hemocytometer. After 21 days, the average count of non-adherent cells present in the type 1

endothelial cell coculture medium was 44,200 ± 13,200; whereas the average count of non-adherent cells present in the type 2 endothelial cell coculture medium was 3,900 ± 750. Thus, a statistically significant ($p<0.01$) increase of hemopoietic cells was observed in the type 1 endothelial cell cocultures as compared to the type 2 endothelial cell cocultures. FIG. 1 illustrates that in an *in vitro* coculture, unactivated type 1 endothelial cells (●) can maintain stem cells for more than 5 weeks, induce the stem cells to proliferate and differentiate into hemopoietic progenitors, and support hemopoiesis for more than 5 weeks (long-term); whereas unactivated type 2 endothelial cells (■) do not support such cell maintenance nor hemopoiesis.

To determine if the heterogenous stages of differentiation and cytokine production of the mobilized (5-FU-induced) stem cells may influence the ability of type 1 endothelial cells or type 2 endothelial cells to maintain the stem cells and support hemopoiesis in a coculture *in vitro*, type 1 endothelial cells and type 2 endothelial cells were also analyzed in coculture with the stem cell subpopulation purified by flow cytometry. Similar results were obtained using these stem cells purified from untreated mice; i.e., in an *in vitro* coculture, unactivated type 1 endothelial cells can maintain stem cells for more than 5 weeks, induce the stem cells to proliferate and differentiate into hemopoietic progenitors, and support hemopoiesis for more than 5 weeks. Unactivated type 2 endothelial cells do not support such cell maintenance nor hemopoiesis. The results, as summarized in Table 4, suggest that pretreatment with 5-FU did not significantly alter the ability of stem cells to respond to type 1 endothelial cells in coculture *in vitro*.

Table 4
Non-adherent cells (10^3/week ± std. error)

Stem cell source	Control	type 1 EC	type 2 EC	Significance*
5-FU induced	0.0 ± 0.0	106 ± 35.2	3.5 ± 2.2	P<0.05
flow cytometry	0.0 ± 0.0	96.0 ± 12.0	0.0 ± 0.0	P<0.01

*statistical significance

The proliferation and differentiation of Lin-, Sca+ cells (isolated by flow cytometry) in the type 1 endothelial cell-stem cell coculture (without adding exogenous cytokines) into hemopoietic progenitors was also evaluated *in vitro*. As shown in FIG. 2A, after 5 days of coculturing the stem cells on monolayers of type 1 endothelial cells ("LEC"), a small number of hematopoietic foci (HC) have appeared. The size of these foci gradually increase in time as illustrated in FIG. 2B. In FIG. 2B, illustrated are cells after 3 weeks of coculture, wherein the cobblestone areas are areas indicative of active hemopoiesis. Cytospin preparations of the non-adherent cells were stained with Giemsa solution for morphologic analysis. Aliquots of the non-adherent cells were incubated with conjugated monoclonal antibodies against murine cell surface receptors including B220, Gr-1, MAC-1, Lyt-2, Ly-1, L3T4, c-mpl, and ter119 for pheno-typical analysis by flow cytometry (with data collection and analysis of fluorescent intensities). The main phenotype of the non-adherent cells in the type 1 endothelial cell-stem cell cocultures consisted of hemopoietic progenitors wherein 47% were of the monocyte/macrophage lineage (MAC-1+), 33% were of the granulocyte lineage (Gr-1+), 16% were of the megakaryocyte lineage (c-mpl+), and present were low percentages (<5%) of erythroblasts (ter119+) and B cells (B220+).

EXAMPLE 4

In another illustration of ability of type 1 endothelial cells to support the maintenance and growth of hemopoietic stem cells (without addition of exogenous cytokines), analyzed were the number of stem cells adhering to the type 1 endothelial cell monolayer or the type 2 endothelial cell monolayer after long-term coculture *in vitro*. After 6 to 8 weeks, the cocultures were terminated and the adherent layer was removed gently with scraping, and the cells of the adherent layer were assayed for colony forming cells (CFC) using the fibrin clot assay described in Example 5 (see also, Bruno et al., 1991, *Blood*, 77:2339-46). Thus, the number of CFC reflected the number of hemopoietic stem cells (from the 5-FU induced subpopulation) present in the adherent cell fraction of the respective cocultures. As a control, adherent cells from bone marrow cultures (BMC) were cultured on collagen precoated wells. The adherent cell fraction from the respective cocultures were harvested after 6 weeks of coculture, and then cultured in a fibrin clot system in the presence of SCF and IL-3. After 7 days of culture, the number of CFC (defined as a colony containing >40 cells) were counted. The results, shown in Table 5, are the mean ± standard error, with the statistical significance estimated by the Student's t-test (P values >0.05 were considered statistically non significant).

Table 5
CFC (10^5 ± std. error)

	Control	type 1 EC	type 2 EC	signifi-cance*
BMC	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	-
5-FU induced	0.0 ± 0.0	69.0 ± 3.5	0.5 ± 0.0	P<0.05

*statistical significance

Table 5 illustrates that in an *in vitro* coculture, unactivated type 1 endothelial cells can maintain stem cells long-term (e.g., for more than 6 weeks), and those stem cells can be induced to proliferate and differentiate into hemopoietic cells capable of forming colonies (CFC).

EXAMPLE 5

In another illustration of ability of type 1 endothelial cells to support the proliferation of hemopoietic stem cells into hemopoietic progenitors, analyzed were the ability of type 1 endothelial cells, as compared to type 2 endothelial cells, to support the survival and proliferation of specific hemopoietic progenitors. The non-adherent cells from type 1 endothelial cell-stem cell coculture and from type 2 endothelial cell-stem cell cocultures (each in the absence of exogenous cytokines) were assayed in a fibrin clot assay to detect granulocyte/macrophage (CFU-GM), erythroid (burst forming unit, BFU-E), megakaryocytic (CFU-Mk) and colony forming cells (CFC) using methodology described previously (Kuriya et al., 1989, *Exp. Hematol.* 15:896-901).

Briefly, the non-adherent cells harvested from the respective cocultures were placed in 20% bovine fibrinogen and 10% human plasma thrombin ($1x10^4$ cells/clot) in the presence of different cytokines (SCF, IL-3, IL-1α, and erythropoietin (Epo)). Aliquots of 0.4 ml were placed in the center of 35 mm culture dishes. Once the fibrin clots had formed, the clots were bathed with 1 ml of serum-free medium (X-Vivo; BioWhittaker, Inc.). The dishes were then incubated at 37°C in a humidified atmosphere flushed with 5% CO_2. After seven days of culture, the number of CFC, CFU-GM, CFU-Mk, and BFU-E were evaluated. For evaluation, the number of CFC (>40 cells) were counted in the non-adherent cell cultures treated with SCF and IL-3. The fibrin clot was dried and stained for acetylcholinesterase (AChE) activity to identify CFU-Mk (Jackson, 1973, *Blood*, 42:413-

421). Groups of three or more AChE(+) cells clustered together were scored as CFU-Mk. Cultures treated with IL-23 and Epo were dried and stained with benzidine to identify and count BFU-E colonies. Cultures treated with GM-CSF were counted for CFU-GM colonies. As shown in FIG. 3A, BFU-E progenitors were observed in the non-adherent cells from the beginning of the type 1 endothelial cell-stem cell coculture, and reaches peak production during the fourth week of coculture. This erythropoiesis continued until this study was terminated. In contrast, the type 2 endothelial cell-stem cell coculture did not support the survival of BFU-E. As shown in FIG. 3B, CFU-GM progenitors were also observed in the non-adherent cells from the beginning of the type 1 endothelial cell-stem cell coculture, and reaches peak production during the third week of coculture. As shown in FIG. 3C, CFU-Mk progenitors were also observed in the non-adherent cells throughout the experimental period of the type 1 endothelial cell-stem cell coculture, and reaches peak production during the second week of coculture.

EXAMPLE 6

This example illustrates that the ability of type 1 endothelial cells to sustain stem cell growth and maintenance, and to support the proliferation of hemopoietic stem cells into hemopoietic progenitors, in *an in vitro* coculture is due to the presence of factors secreted by the type 1 endothelial cells in coculture. To demonstrate this, the medium of either type 1 endothelial cell cultures or type 2 endothelial cell cultures (in the absence of added exogenous cytokines) was investigated for the possible presence of soluble factors which could support the maintenance and proliferation of stem cells and promote *in vitro* hemopoiesis. Briefly, type 1 endothelial cells and type 2 endothelial cells were grown in separate cultures to 90% confluence, wherein the culture supernatant was removed, 10 mls of serum-free culture medium was added, and the cells

were cultured overnight. Then, the medium from each culture was removed ("conditioned medium"), centrifuged at 2,000 x g for 15 minutes, and passed through a 0.2 μm filter before use to ensure that no cells were present. The capacity of either type 1 endothelial cell conditioned medium or type 2 endothelial cell conditioned medium to promote hemopoiesis from normal bone marrow cells was assayed using the fibrin clot system described in Examples 5 and 6 herein. Briefly, after 7 days of culture between the respective conditioned medium and normal bone marrow cells in the fibrin clot system, the number of hemopoietic CFC was evaluated according to their morphology (e.g., compact colonies with >40 cells/colony) and by their cell content. As controls, normal bone marrow cells were cultured with medium alone. The results, shown in Table 6, are the mean ± standard error, with the statistical significance estimated by the Student's t-test (P values >0.05 were considered statistically non significant).

Table 6
CFC (10^5 ± std. error)

	Control	type 1 EC medium	type 2 EC medium	Signifi-cance*
BMC	0.0 ± 0.0	69.0 ± 3.5	0.5 ± 0.5	P<0.001

*statistical significance

Table 6 illustrates that type 1 endothelial cell conditioned medium contains soluble factors which can maintain stem cells in an *in vitro* long-term coculture; and induce those stem cells to proliferate and differentiate into hemopoietic cells capable of forming colonies (CFC). Further, the soluble factors are produced by the type 1 endothelial cells.

EXAMPLE 7

This example illustrates characterization of activated type 1 endothelial cells and activated type 2 endothelial cells. It was determined during the development of the present invention that type 1 endothelial cells can be activated by cytokines (growth factors), thereby causing the activated type 1 endothelial cells to secrete growth factors that may interact with the hemopoietic stem cells in a hematopoietic microenvironment. Cytokines causing the activation of type 1 endothelial cells have been identified herein to include tumor necrosis factor alpha (TNF-α), IL-1β, and IFN-γ (acting separately, and also synergistically when in combination). To illustrate such activation, subpopulations of unactivated type 1 endothelial cells and of unactivated type 2 endothelial cells were activated by TNF-α, and then characterized and compared for cytokine expression. Each subpopulation was isolated and purified using the methods according to Example 1. Cultures of each subpopulation were treated for four hours with 25 ng/ml TNFα at 37°C. The TNFα was then removed by washing the TNFα-activated cells. As controls, in parallel experiments, respective cytokine expression was quantitated in unactivated type 1 endothelial cells and unactivated type 2 endothelial cells. The activated endothelial cells were then evaluated by commercially available ELISAs for quantitating the expression of cytokines including IL-1β, IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN-γ, and TNF-α.

FIG. 4 illustrates the comparison of cytokine expression (measured as pg/ml) between unactivated type 1 endothelial cells (FIG. 4A), unactivated type 2 endothelial cells (FIG. 4B), type 1 endothelial cells activated by TNF-α (FIG. 4C), and type 2 endothelial cells activated by TNF-α (FIG. 4D). Similar cytokine expression was observed when type 1 endothelial cells were activated with either IL-1β or IFN-γ. It is interesting to note that IL-6 (TNFα-induced IL-6) is a signal for cell proliferation that organ cells

use in regenerative processes (see, e.g., liver regeneration, Michalopoulos and DeFrances, 1997, *Science* 276:60-66).

The activated type 1 endothelial cells and the activated type 2 endothelial cells were also analyzed for NO production. Nitric oxide (NO) is a molecule formed in cells from L-arginine residues by the enzyme nitric oxide synthase (NO synthase). Prolonged exposure to nitrous oxide has been shown to impair a hematopoietic microenvironment as well as to impair the maintenance of hemopoietic stem cells (Suzuki et al., 1990, *Anesth. Analg.* 71:389-93; Konno, 1991, *Masui* 40:702-12). Induction of NO in mammalian vessel endothelial cells has also been shown (Shuler et al., 1995, *J. Leukoc. Biol.* 57:116-21; Inoue et al., 1995, *Arteriorscler. Thromb. Vasc. Biol.* 15:1255-61).

To investigate whether subpopulations of activated type 1 endothelial cells and of activated type 2 endothelial cells produce NO, each subpopulation was activated by either TNF-α, IFN-γ, or a combination of TNF-α and IFN-γ, and then characterized and compared for NO expression. Each subpopulation was isolated and purified using the methods according to Example 1. Cytokine induced-activation was performed using the methods herein. The supernatants from type 1 endothelial cell cultures and from type 2 endothelial cell cultures were assayed for NO production according to previously described methods for determining NO (Kolb et al., 1994, *J. Biol. Chem.* 269:9811-9816; Viinikka, 1996, *Scand. J. Clin. Lab. Invest.* 56:577-81). Briefly, cell-free supernatants (50 µl) were transferred into wells of a 96 well microtiter plate containing per well 100 µl Griess reagent (1% sulfanilamide in 30% acetic acid and 0.5% of N-1-naphtylethylenediamine dihydrochloride in 60% acetic acid). The mixture was incubated for 10 minutes at 25°C, and the product of the reaction was detected by absorbance at 540 nm using a precision microplate reader. The concentration of nitrite was calculated by comparison with standard

concentrations of sodium nitrite. Concentrations of nitrite/nitrate are typically used in the art as quantitative indices of NO production.

FIG. 5 illustrates the comparison of NO production (measured as NO_2 in micromoles- μM) in unactivated type 1 endothelial cells (control □), unactivated type 2 endothelial cells (control ■), type 1 endothelial cells activated by TNF-α (TNF-α □), type 2 endothelial cells activated by TNF-α (TNF-α ■), type 1 endothelial cells activated by IFN-γ (IFN-γ □), type 2 endothelial cells activated by IFN-γ (IFN-γ ■), type 1 endo-thelial cells activated by a combination of TNF-α and IFN-γ (TNF-α + IFN-γ □), and type 2 endothelial cells activated by TNF-α and IFN-γ (TNF-α + IFN-γ ■). As shown in Fig. 5, very little NO_2 is constitutively produced in either unactivated type 1 endothelial cells or unactivated type 2 endothelial cells. Also, little induction (as measured by NO_2) is observed in type 1 endothelial cells or type 2 endothelial cells when activated by either TNF-α or by IFN-γ. In contrast, activation of type 2 endothelial cells by a combination of TNF-α and IFN-γ resulted in significant amounts (e.g., > 50μM) of NO_2 produced by such activated type 2 endothelial cells.

Using the methods described herein, an *in vitro* coculture of activated type 1 endothelial cells and stem cells, and an *in vitro* coculture of activated type 2 endothelial cells and stem cells were analyzed for the ability to maintain the stem cells, induce the stem cells to proliferate and differentiate into hemopoietic progenitors, and support long-term hemopoiesis. The results, as summarized in Table 7, suggest that activated type 1 endothelial cells appear to perform at least as well as unactivated type 1 endothelial cells in the ability to induce maintenance and proliferation of stem cells in coculture *in vitro* (e.g., as compared to the results in Table 4).

Table 7
Non-adherent cells (10^3/week ± std. dev.) in a coculture containing TNFα-activated endothelial cells

Stem cell source	Control	type 1 EC	type 2 EC	Signifi-cance*
5-FU induced	0.0 ± 0.0	319 ± 146.2	0.6 ± 0.4	P<0.05

*statistical significance

Therefore, the inability of type 1 endothelial cells to produce significant amounts of nitric oxide may be an additional factor important in providing a hematopoietic microenvironment in which hemopoietic stems cells can be maintained, and be induced to proliferate and differentiate into hemopoietic progenitors.

From the foregoing, it will be obvious to those skilled in the art that various modifications in the above-described methods, and compositions can be made without departing from the spirit and scope of the invention. Accordingly, the invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. Present embodiments and examples, therefore, are to be considered in all respects as illustrative and not restrictive, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

What is claimed is:

1. A cell culture method comprising coculturing *in vitro* hemopoietic stem cells in the presence of a composition which provides a microenvironment for maintaining and inducing proliferation of the stem cells, wherein the composition comprises a substantially pure population of type 1 endothelial cells or type 1 endothelial cell conditioned medium.

2. The method of claim 1, wherein the composition further provides a microenvironment which directs the stem cells to differentiate into hemopoietic progenitor cells.

3. The method of claim 1, wherein the composition comprises a substantially pure population of type 1 endothelial cells of human origin.

4. The method of claim 3, wherein said type 1 endothelial cells are selected from the group consisting of immortalized type 1 endothelial cells, type 1 endothelial cells pretreated with TNFα, type 1 endothelial cells pretreated with IFN-γ, and type 1 endothelial cells pretreated with both TNFα and IFN-γ.

5. The method of claim 1 wherein the stem cells are human stem cells.

6. The method according to claim 5, wherein the stem cells are derived from bone marrow.

7. The method according to claim 5, wherein the stem cells are collected from peripheral blood or cord blood.

8. The method of claim 1, wherein the type 1 endothelial cells and stem cells are allogeneic.

9. The method of claim 1, wherein the type 1 endothelial cells and stem cells are autologous.

10. The method of claim 1, wherein the coculture is in the presence of serum-free medium and in the absence of exogenous cytokines.

11. The method of claim 1, wherein the stem cells comprise transfected stem cells.

12. The method of claim 1, wherein the composition comprises said type 1 endothelial cells, and wherein the stem cells are cocultured in direct physical contact with the type 1 endothelial cells.

13. The method of claim 12, wherein the stem cells adhere to a monolayer of the type 1 endothelial cells.

14. The method of claim 13, further comprising separating the adherent stem cells from the monolayer by a means selected from the group consisting of physical means, and enzymatic means.

15. The method of claim 1, wherein the composition comprises said type 1 endothelial cells, and wherein the stem cells are cocultured in a non-contacting relationship with the type 1 endothelial cells.

16. The method of claim 15 wherein the non-contacting relationship comprises a first support surface onto which is grown the stem cells, and a second support surface onto which is grown the type 1 endothelial cells.

17. The method of claim 16, wherein the first support surface is on a separate support matrix than the second support surface.

18. The method of claim 17, wherein the first support surface is a surface on a microporous membrane.

19. The method according to claim 16 wherein the first support surface comprises one side of a support matrix, and the second support surface comprises the other side of the same support matrix.

20. The method of claim 19, wherein the support matrix is a microporous membrane.

21. The method according to claim 2, wherein the hemopoietic progenitors are present in coculture medium.

22. The method according to claim 21, further comprising harvesting the hemopoietic progenitors from the coculture medium.

23. The method of claim 1, wherein the composition comprises type 1 endothelial cell conditioned medium which perfuses the stem cells.

24. The method of claim 2, wherein the composition comprises type 1 endothelial cell conditioned medium which perfuses the stem cells.

25. The method of claim 24, wherein the hemopoietic progenitors are present in coculture medium.

26. The method of claim 25, further comprising harvesting the hemopoietic progenitors from the coculture medium.

27. The method of claim 23, wherein the type 1 endothelial cell condition medium is exchanged for coculture medium at fixed intervals.

28. A method for obtaining *ex vivo* human progenitor cells comprising coculturing *in vitro* human hemopoietic stem cells in the presence of a composition which provides a microenvironment for maintaining and inducing proliferation of the stem cells, and differentiation of the stem cells into the progenitor cells, wherein the composition comprises a substantially pure population of type 1 endothelial cells or type 1 endothelial cell conditioned medium.

29. The method of claim 28, wherein the hemopoietic progenitors are present in coculture medium.

30. The method of claim 29, further comprising harvesting the hemopoietic progenitors from the coculture medium.

31. The method of claim 28, wherein the type 1 endothelial cells and stem cells are allogeneic.

32. The method of claim 28, wherein the type 1 endothelial cells and stem cells are autologous.

33. The method of claim 28, wherein the coculture is in the presence of serum-free medium and in the absence of exogenous cytokines.

34. The method of claim 28, wherein the composition comprises a substantially pure population of type 1 endothelial cells of human origin.

35. The method of claim 28, wherein the composition comprises type 1 endothelial cell conditioned medium which perfuses the stem cells.

36. The method of claim 34, wherein said type 1 endothelial cells are selected from the group consisting of immortalized

type 1 endothelial cells, type 1 endothelial cells pretreated with TNFα, type 1 endothelial cells pretreated with IFN-γ, and type 1 endothelial cells pretreated with both TNFα and IFN-γ.

37. A method for obtaining *ex vivo* human stem cell proliferation, differentiation, or both, comprising coculturing *in vitro* human hemopoietic stem cells in the presence of a composition which provides a microenvironment for maintaining and inducing proliferation of the stem cells, wherein the composition comprises a substantially pure population of type 1 endothelial cells or type 1 endothelial cell conditioned medium.

38. The method of claim 37, further comprising harvesting the stem cells generated from the coculture.

39. The method of claim 37, wherein the type 1 endothelial cells and stem cells are allogeneic.

40. The method of claim 37, wherein the type 1 endothelial cells and stem cells are autologous.

41. The method of claim 37, wherein the coculture is in the presence of serum-free medium and in the absence of exogenous cytokines.

42. The method of claim 37, wherein the composition comprises a substantially pure population of type 1 endothelial cells of human origin.

43. The method of claim 37, wherein the composition comprises type 1 endothelial cell conditioned medium which perfuses the stem cells.

44. The method of claim 42, wherein said type 1 endothelial cells are selected from the group consisting of immortalized type 1 endothelial cells, type 1 endothelial cells pretreated with TNFα, type 1 endothelial cells pretreated with IFN-γ, and type 1 endothelial cells pretreated with both TNFα and IFN-γ.

45. A composition useful in the method of claim 1, wherein the composition comprises human type 1 endothelial cells selected from the group consisting of immortalized type 1 endothelial cells, type 1 endothelial cells pretreated with TNFα, type 1 endothelial cells pretreated with IFN-γ, and type 1 endothelial cells pretreated with both TNFα and IFN-γ.

46. A composition useful in the method of claim 28, wherein the composition comprises human type 1 endothelial cells selected from the group consisting of immortalized type 1 endothelial cells, type 1 endothelial cells pretreated with TNFα, type 1 endothelial cells pretreated with IFN-γ, and type 1 endothelial cells pretreated with both TNFα and IFN-γ.

47. A composition useful in the method of claim 37, wherein the composition comprises human type 1 endothelial cells selected from the group consisting of immortalized type 1 endothelial cells, type 1 endothelial cells pretreated with TNFα, type 1 endothelial cells pretreated with IFN-γ, and type 1 endothelial cells pretreated with both TNFα and IFN-γ.

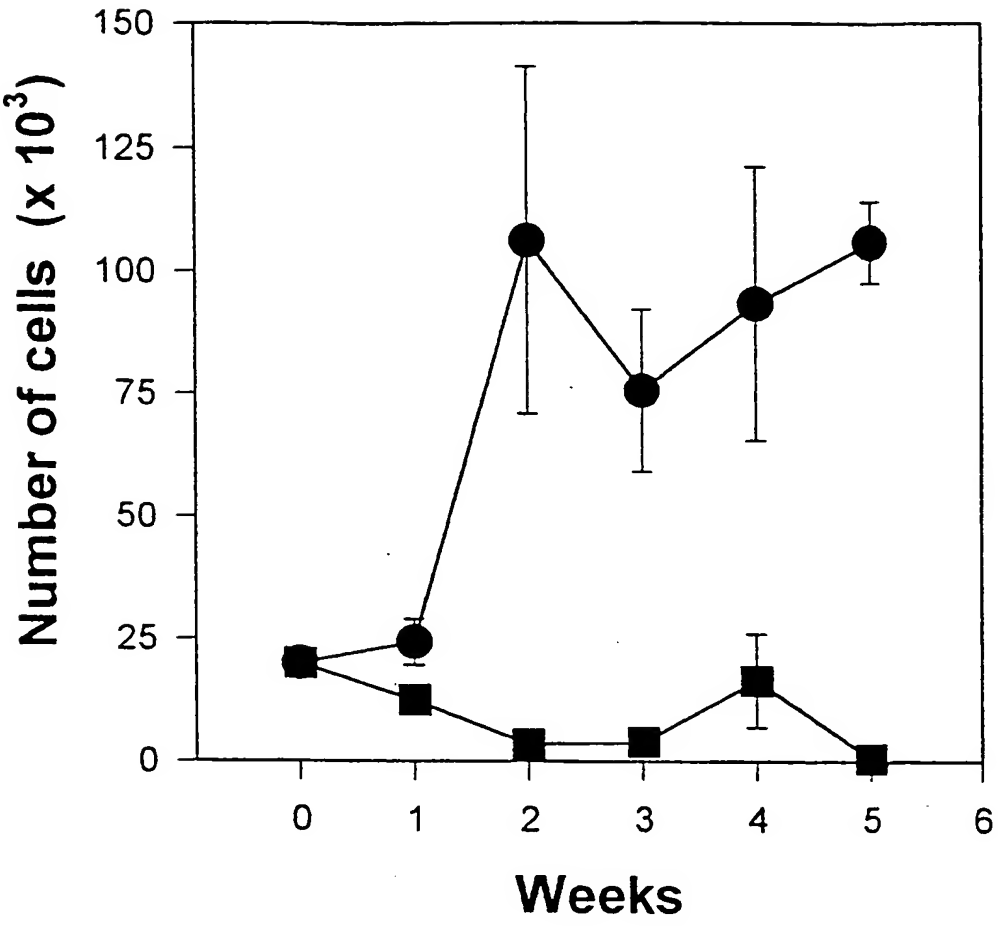


FIG. 1

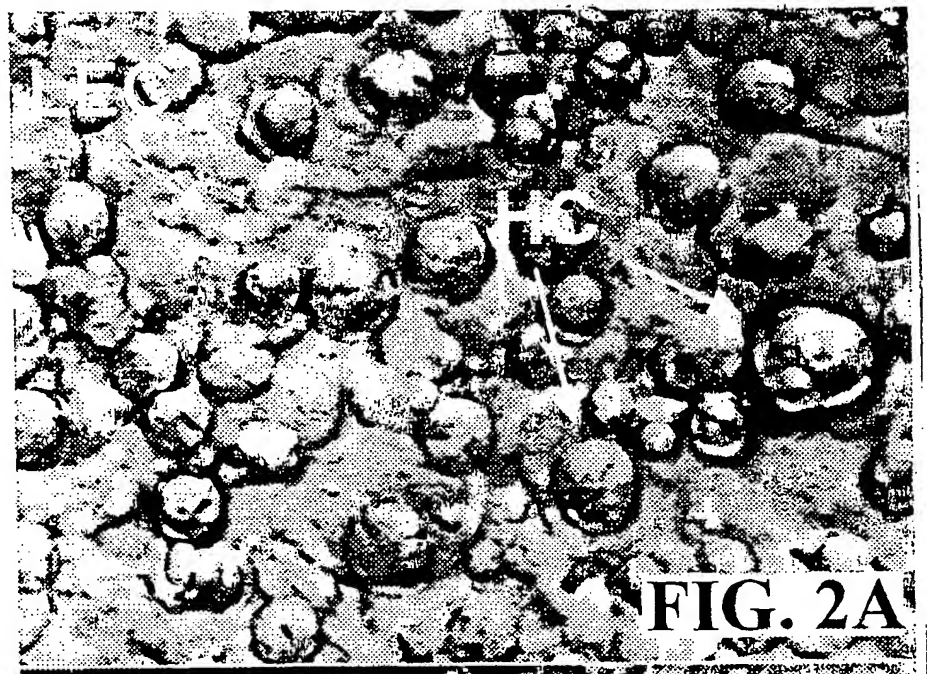


FIG. 2A

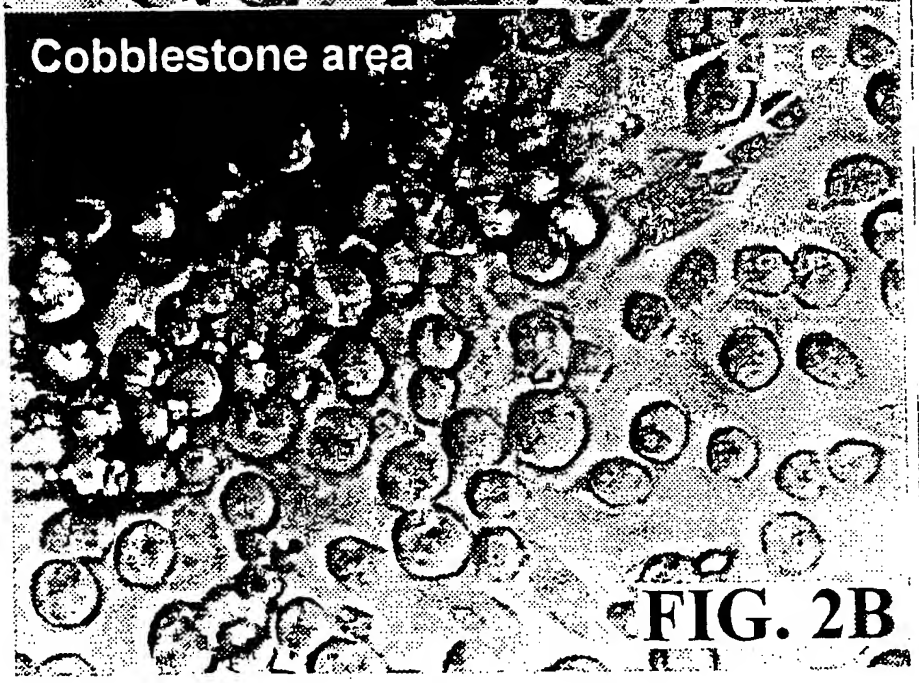


FIG. 2B

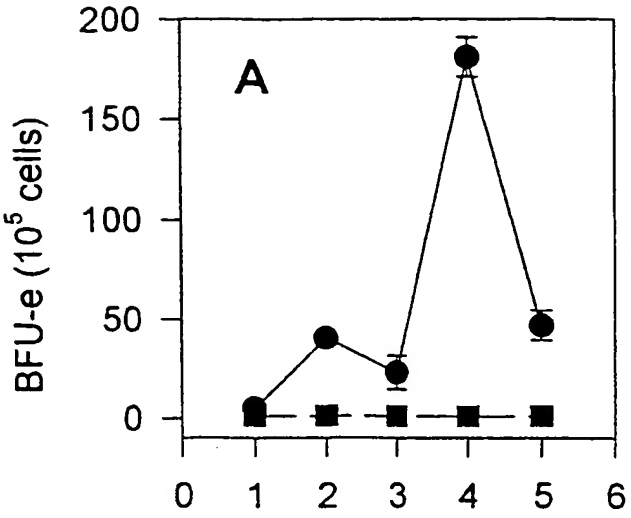


FIG. 3A

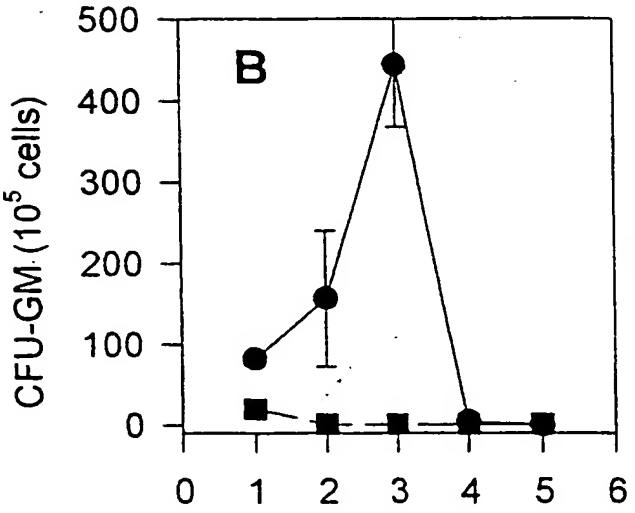


FIG. 3B

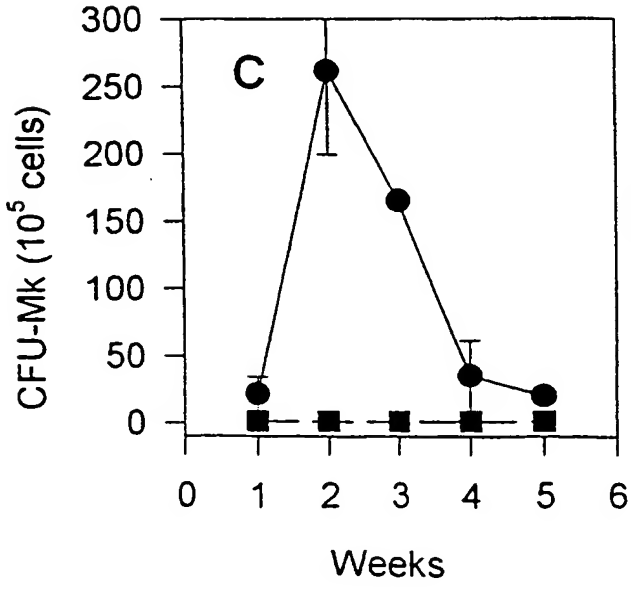


FIG. 3C

FIG. 4A

LEC-1

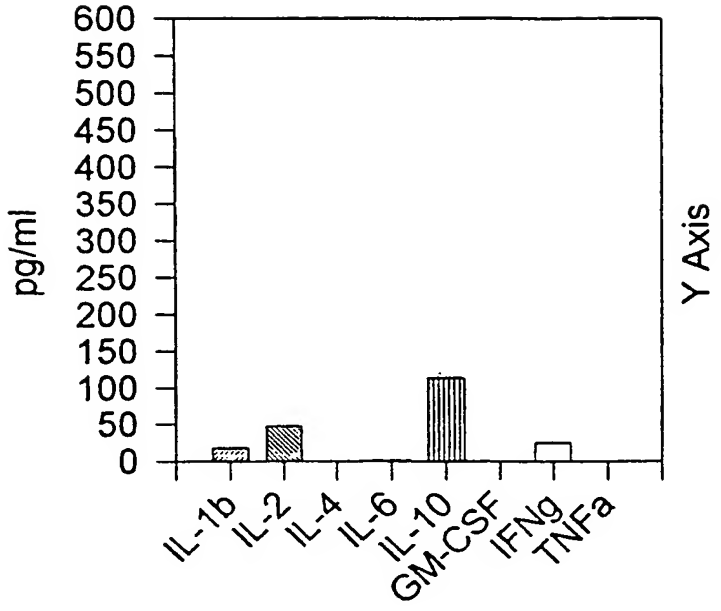
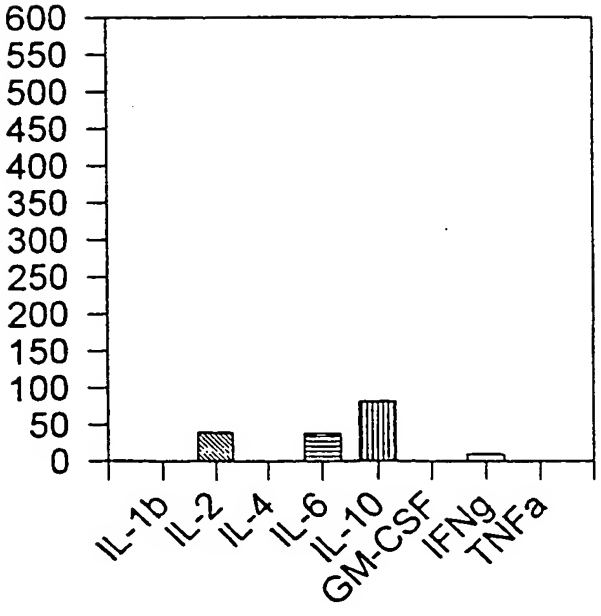


FIG. 4B

LEC-2



LEC-1 +TNFα

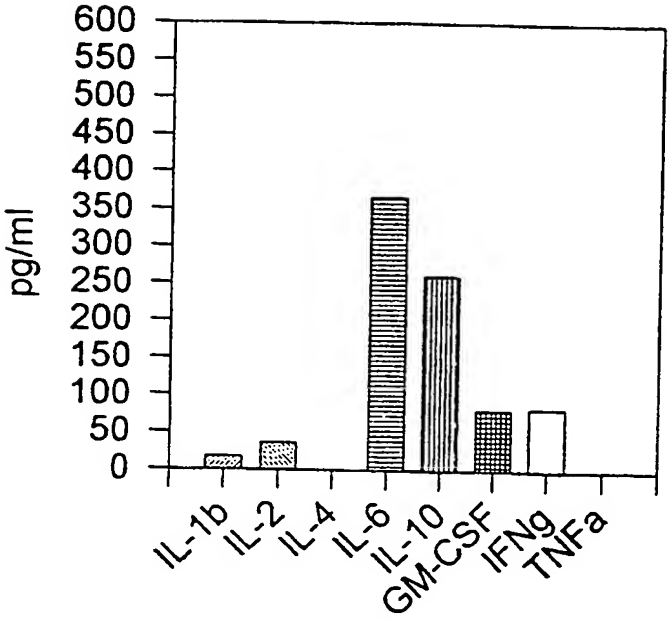


FIG. 4C

LEC-2 +TNFα

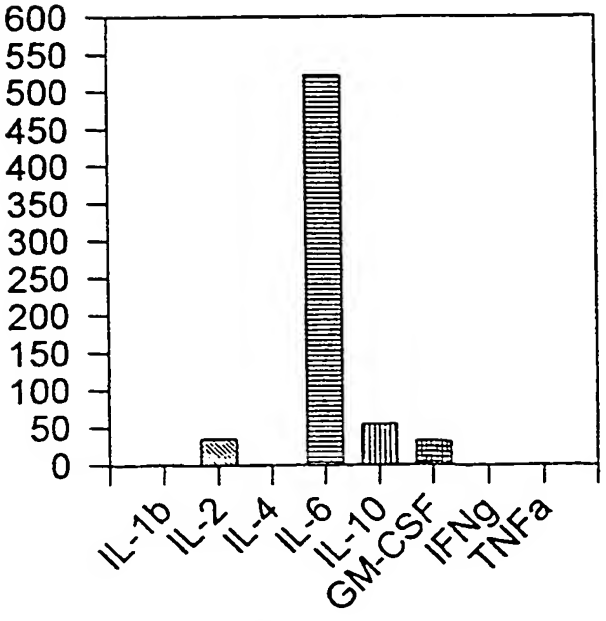


FIG. 4D

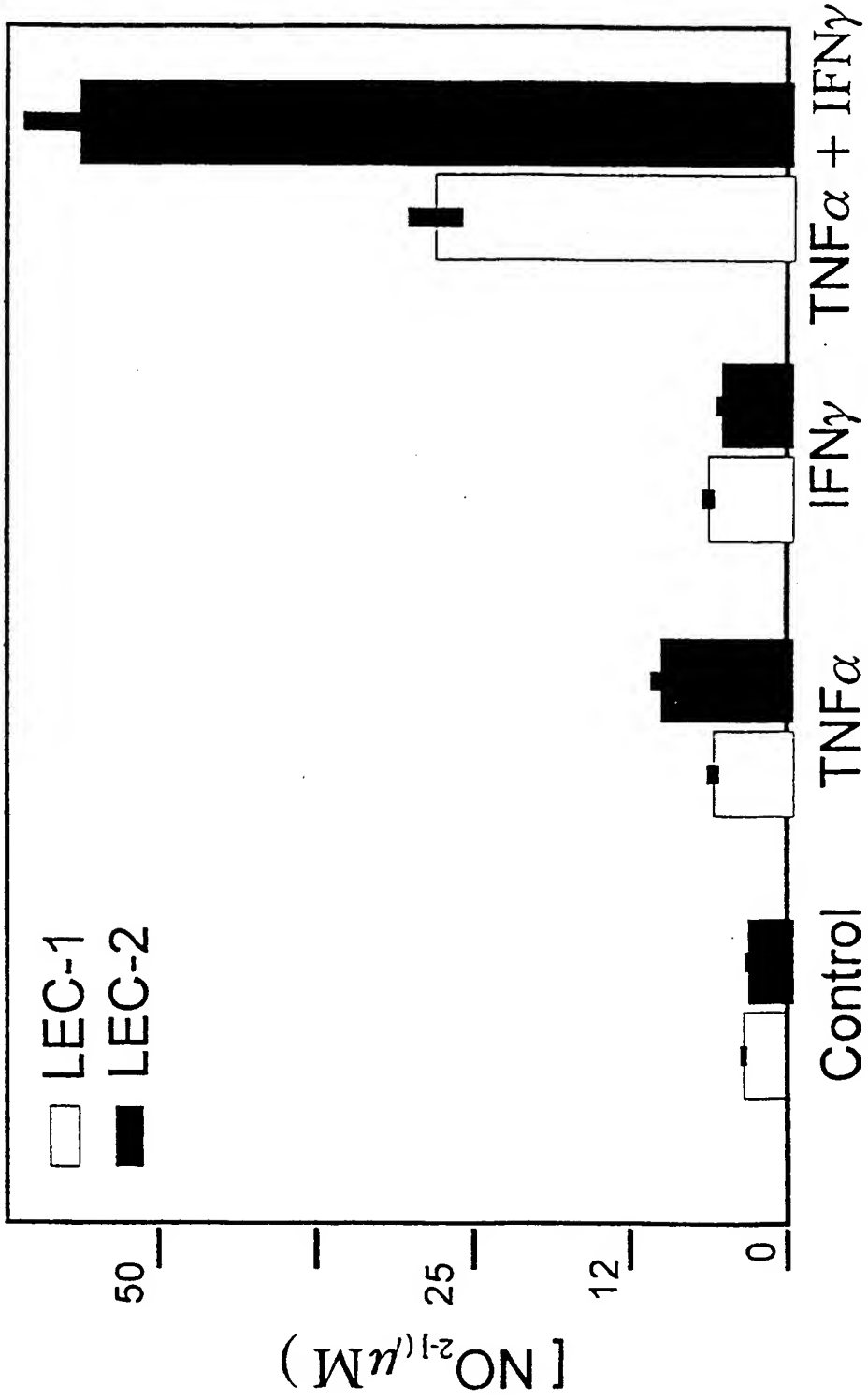


FIG. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/12996

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/00, 5/02, 5/06, 5/08
US CL : 435/325, 347, 366, 372, 373, 378, 379, 395, 401

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/325, 347, 366, 372, 373, 378, 379, 395, 401

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE, BIOSIS, WPIDS, CAPLUS
search terms: type I endothelial, hematopoietic stem cells, stem cells, coculture, stroma, stromal, precursor, conditioned medium

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	RAFII. S. et al. Human Bone Marrow Microvascular Endothelial Cells Support Long-Term Proliferation and Differentiation of Myeloid and Megakaryocytic Progenitors. 01 November 1995. Vol. 86. No. 9. pages 3353-3363, especially pages 3354-3357.	1-47
Y	WO 95/19793 A (THE UNITED STATES OF AMERICA, represented by THE SECRETARY OF THE NAVY) 27 July 1995, pages 1-60, especially pages 4-6, 9, 11-14, and 20.	1-47

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
12 OCTOBER 1998

Date of mailing of the international search report
29 OCT 1998

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